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Assessment of Differential Gene Expression Patterns in Human Colon Cancers

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Objective
To use a novel genomic approach to determine differential gene expression patterns in colon cancers of different metastatic potential.

Summary Background Data
Colorectal cancer is the third leading cause of cancer deaths in the United States; despite aggressive treatment strategies, the 5-year survival rate for metastatic cancer has not changed in 50 years. The analysis of changes in gene expression patterns associated with metastasis may provide new treatment strategies.

Methods
Human colon cancer cells KM12C (derived from a Dukes B colon cancer), KML4A (a metastatic variant derived from KM12C), and KM20 (derived from a Dukes D colon cancer) were extracted for RNA. In addition, RNA was extracted from normal colon, primary cancer, and liver metastasis in a patient with metastatic colon cancer. Gene expression patterns for approximately 1,200 human genes were analyzed and compared by cDNA array techniques.

Results
Of the roughly 1,200 genes assessed in the KM cell lines, 9 genes were noted to have a more than threefold change in expression (either increased or decreased) in the more metastatic KML4A and KM20 cells compared with KM12C. Assessment of tissues from a patient with metastatic colon cancer demonstrated a more than threefold change in the expression of 14 genes in the primary cancer and liver metastasis compared with normal mucosa.

Conclusions
Using cDNA expression array technology, the authors identified genes with expression levels that are altered with metastasis. The ability to analyze and compare the expression patterns of multiple genes simultaneously provides a powerful technique to identify potential molecular targets for novel therapeutic strategies.

Colorectal cancer is a significant health problem worldwide. Approximately 130,000 new cases are anticipated in the Unites States alone in 2000.[1] The death rate remains third to lung and prostate cancer in men and lung and breast cancer in woman, with approximately 57,000 deaths expected to occur this year.[1] Although patients may undergo surgical resection for possible cure, approximately 50% of patients die of their disease secondary to metastasis noted either at the time of the initial resection or months to years later.[1] Current chemotherapy regimens have been ineffective; therefore, a better understanding of the molecular events leading to tumor metastasis is crucial to the development of antineoplastic therapies.

The genetic changes associated with colorectal carcinogenesis have been well characterized. Current research supports the notion that most, if not all, colorectal cancers arise from preexisting benign polyps. This adenoma–carcinoma sequence, initially proposed by Fearon and Vogelstein,[2] involves multiple genetic events that eventually culminate in neoplasia. The serial genetic changes that account for this neoplastic transformation include inactivation of tumor suppressor genes (e.g., APC, DCC, p53) by mutation, deletion, or loss of heterozygosity and activation of oncogenes (e.g., K-ras).[3] In addition, other factors, such as DNA hypomethylation, likely contribute to the neoplastic process.[4,5] In

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marked contrast, relatively little is known about the genetic changes associated with colon cancer metastasis.

The metastasis of cancer cells requires the development of a complex phenotype that is characterized by a transient or permanent change in gene expression. This process favors the survival and growth of a small subpopulation of cells that preexist within a heterogeneous primary neoplasm. Abnormal gene expression by cancer cells leads to morphologic changes that benefit cells in various survival characteristics. To produce metastases, tumor cells must succeed in invasion, embolization, survival in the circulation, arrest in a distant capillary bed, and extravasation into and multiplication in organ parenchyma (e.g., liver or lung).[6,7] An important aspect of this process is the ability of cancer cell metastases to evade immune detection in the circulation. This "immune escape" phenomenon is thought to occur, in part, by novel proteins of the tumor necrosis factor (TNF) family.[8–10] These proteins include TNF-related apoptosis-inducing ligand (TRAIL) and FasL, which interact with cell surface TNF receptors (TNFRs) to induce programmed cell death.[11–13] Both TRAIL and FasL function as inducers of apoptosis in many cellular events such as autoimmunity, activation-induced cell death, and immune privilege.[14–16] In addition, it appears that both proteins may play an important role in the escape of certain cancer cells from surveillance and therefore contribute significantly to the metastasis of these cancers.[8–16]

The purpose of our study was to analyze alterations in gene expression patterns (either increased or decreased) associated with colon cancer metastasis. We used a novel genomic approach (cDNA expression arrays) to assess the expression of approximately 1,200 genes in human colon cancers of varying metastatic potential as well as normal colonic mucosa, primary cancer, and a liver metastasis resected from a patient with Dukes D colon cancer. Using this technology to focus on genetic mediators of cancer metastasis, new insights may be obtained that ultimately could lead to the development of novel therapies for the treatment and possible prevention of metastatic colorectal cancer.

METHODS

Materials

Tissue culture media and reagents were obtained from Gibco-BRL (Grand Island, NY). Total RNA was isolated using RNAzol (Biotex, Houston, TX) and digested with RNase free DNase I (Clontech Laboratories Inc., Palo Alto, CA). [α-^{32}P]dATP (25 μCi) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The Atlas Human array 1.2 and specific sequence primers for reverse transcription–polymerase chain reaction (RT-PCR) were purchased from Clontech. cDNA probes were synthesized for Northern blots using RT-PCR products and a random labeling kit from Stratagene (La Jolla, CA). Nitrocellulose filters were purchased from Sartorius (Göttingen, Germany). The constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was obtained from Ambion (Austin, TX) and used to ensure both equal loading and the integrity of the RNA samples analyzed by Northern blot. The concentrated protein assay dye was purchased from Bio-Rad Laboratories (Hercules, CA). Immobilon-P nylon membranes for Western blots were purchased from Millipore (Bedford, MA), and x-ray film was purchased from Eastman Kodak (Rochester, NY). The enhanced chemiluminescence system for Western immunoblot analysis was obtained from Amersham (Arlington, Heights, IL). The human anti-TRAIL and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Tissue Procurement

The human colon cancer cell lines KM12C, KML4A, and KM20 were provided by Dr. Isaiah J. Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX).[17] The KM12C cell line was derived from a patient with Dukes B (nonmetastatic) colon cancer. KML4A was derived from KM12C and has been "trained" to metastasize to the liver by multiple (four) rounds of injection into nude mice. The KM20 cell line was derived from a patient with Dukes D (metastatic to the liver) colon cancer. The cells were cultured in MEM medium supplemented with 10% FCS, 1% sodium pyruvate, 1% nonessential amino acids, and 1% MEM essential vitamin mixture.

Samples of primary colon adenocarcinoma, adjacent (5–10 cm from the cancer) normal mucosa, and a liver metastasis were obtained from a patient undergoing elective surgical resection at the University of Texas Medical Branch. Tissue acquisition and subsequent use were approved by the institutional review board.

RNA Isolation and cDNA Microarray Hybridization

Total RNA was extracted from cultured cell lines with RNAzol as recommended by the manufacturer. RNA was digested with RNase free DNase I for 30 minutes at 37°C in 50 mmol/L Tris buffer (pH 6.5) with 10 mmol/L $MgCl_2$ and 10 mmol/L dithiothreitol (DTT). The reaction was terminated with 10× termination mix (0.1 M EDTA [pH 8.0] and 1 mg/mL glycogen), and RNA extraction was performed using the phenol method.[18,19] The quality of total RNA was controlled by agarose gel electrophoresis, as demonstrated by the presence of intact ribosomal RNA (28S and 18S bands). Briefly, labeled cDNA probes were synthesized from 3 μg total RNA in the presence of [α-^{32}P] dATP, 10× dNTP mix (5 mmol/L each dCTP, dGTP, dTTP), human 1.2 10× CDS primer mix, 5× reaction buffer, Moloney murine leukemia virus reverse transcriptase (100 units/μL), and DTT (100 mmol/L), as directed by the manufacturer. Hybridization was then carried out with the Atlas Human array

1.2 nylon membranes overnight at 68°C. Differential gene expression patterns were detected by phosphorimaging, and data were analyzed using the AtlasImage software (Clontech). Arrays were performed in duplicate and an average gene array was generated. Each average array was then used to perform comparative studies to detect differences or similarities between arrays. Gene expression was normalized to overall global gene expression of each array, as recommended by the manufacturer.

Northern Blot Analysis and Probe Preparation

Northern analysis was performed with 30 μg total RNA separated by 1% formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose membrane by standard methods.[20] Blots were then baked, prehybridized, and hybridized as described previously.[21] The cDNA probes for human TRAIL and TNFR2 were generated by RT-PCR. The GAPDH cDNA used in our studies was either generated by RT-PCR or obtained from Ambion. Two μg total RNA was first used in the synthesis of first-strand cDNA, as directed by the manufacturer. Briefly, a 50-μl reaction comprising 2 μg total RNA, 1 μL Oligo (dT), and RNase free water was incubated for 10 minutes at 70°C. The reaction mixture was then mixed with 5× First-Strand Buffer, 0.1 mol/L DTT, and 10 mmol/L dNTP mix and incubated for 2 minutes at 42°C. This was followed by the addition of Superscript II (200 U/μL) and incubation for 50 minutes at 42°C. The reaction was terminated by increasing the reaction mixture temperature to 70°C for 15 minutes. The cDNA generated was then used for PCR reactions using a RT-PCR kit. Specific sequence primers (forward and reverse) used in this study include TRAIL (forward, 5′-CTTTTCCGGCGGCGTTCATGTCCTTC-3′, reverse, 5′-GTTTCTTCCAGGCTGCTTCCCTTTGTAG-3′); TNFR2 (forward, 5′-GCCACTACACTCCAGCCTGAGC-3′, reverse, 5′-CTGCCCTGTGATGCCAAGGAAGCC-3′), and GAPDH (forward, 5′-TCCACCACCCTGTTGCTGTA-3′, reverse, 5′-ACCACAGTCCATGCCATCAC-3′). The subsequent cDNAs were then labeled by random priming with [α-^{32}P] dCTP. The membrane was washed four times with 0.5× SCC/0.1% SDS at 45°C for 2 hours. The membrane was then dried and exposed to x-ray film with an intensifying screen at −70°C. To correct for RNA loading, the signals were normalized with respect to GAPDH in the same blot.

Protein Preparation, Western Immunoblot, and Immunohistochemistry

Western immunoblot analysis was performed as described previously.[22] Cells were lysed with lysis buffer A (50 nmol/L Tris HCl [pH 7.5], 150 mmol/L NaCl, 0.5 mmol/L Nonidet P-40, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride) and 25 μg/mL each aprotinin, leupeptin, and pepstatin A at 4°C for 30 minutes. Lysates were clarified by centrifugation (10,000*g* for 30 minutes at 4°C), and protein concentrations were determined using the method of Bradford.[22] Briefly, total protein (50 μg) was resolved on a 10% polyacrylamide gel and transferred to Immunoblot-P nylon membranes. Filters were incubated overnight at 4°C in blotting solution (Tris-buffer saline containing 5% nonfat dried milk and 0.1% Tween 20) and then for 3 hours with the primary antibody to human TRAIL. Filters were incubated with a horseradish peroxidase-conjugated antirabbit antibody as a secondary antibody for 1 hour. After four final washes, the immune complexes were visualized using enhanced chemiluminescence detection.

For immunohistochemical studies, KM cells were grown on sterile glass slides overnight at 37°C, washed briefly with phosphate-buffered saline (PBS), fixed for 5 minutes in −10°C methanol and air-dried as described previously.[22] Briefly, slides were incubated for 1 hour in 1.5% normal blocking serum in PBS. After removal of the blocking serum, incubation for 30 minutes with the primary antibody (2.0 μg/mL) or mouse IgG isotype diluted in PBS with 1.5% normal blocking serum was performed. This was followed by a 30-minute incubation with the biotin-conjugated secondary antibody at 1 μg/mL diluted in PBS with 1.5% normal blocking serum. Next, the biotin-labeled cells were incubated for 30 minutes with avidin-biotin enzyme reagent, followed by three washes with PBS. A counterstain with hematoxylin was performed, which was washed immediately. Dehydration was then carried out with soaks in 95% and 100% ethanol twice for 10 seconds each, then xylene three times for 10 seconds. Permanent mounting medium was added, followed by placement of a glass coverslip and observation by light microscopy. Controls for these experiments included preincubation with blocking serum derived from the same species in which the secondary antibody was raised with no primary antibody or with the mouse IgG.

RESULTS

Differential Gene Expression Patterns in the KM Colon Cancer Cell Lines

To understand and identify mediators in the development of metastatic disease, we used cDNA expression arrays to assess the expression pattern of roughly 1,200 human genes simultaneously. After extracting total RNA from the KM cell lines, ^{32}P-labeled cDNA probes were synthesized and hybridized to the Atlas Human 1.2 expression array from Clontech (Fig. 1A). Analysis of the expression arrays demonstrated the presence of 253 (21%), 186 (16%), and 209 (17%) genes in the KM12C, KML4A, and KM20 cell lines, respectively. Each array was performed in duplicate and an average composite array was developed for each KM cell line. The composite arrays of the three KM cell lines were then compared.

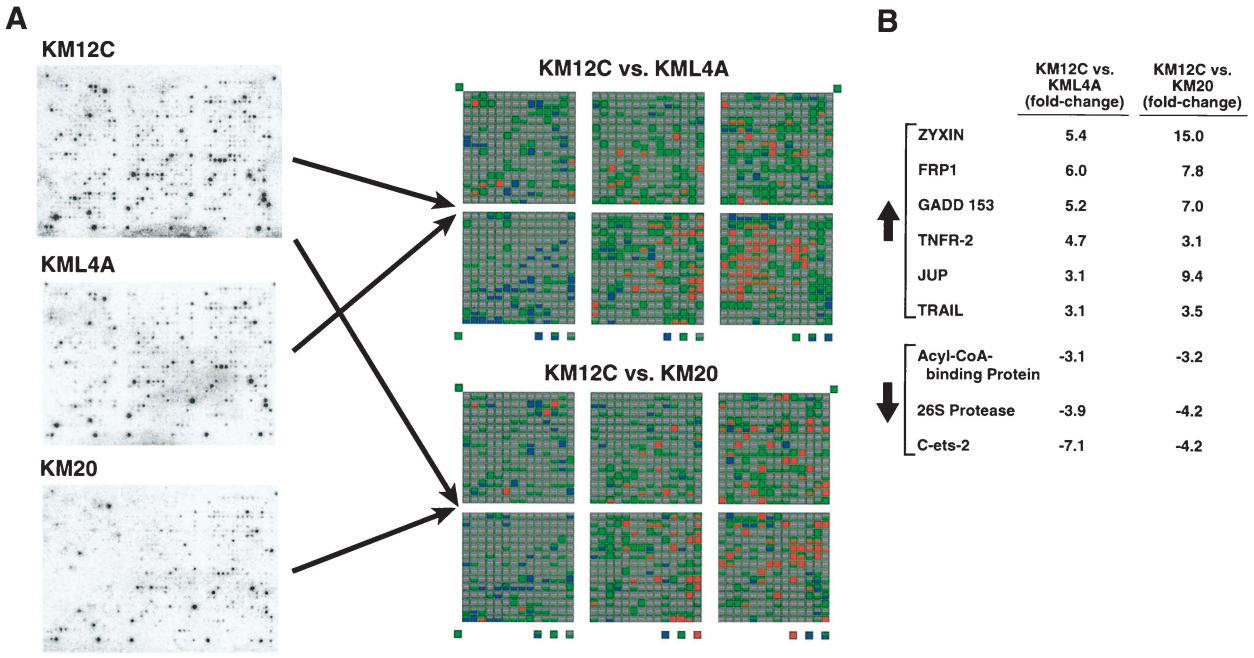

Figure 1. Gene expression profile in KM colon cancer cells. (**A**) ^{32}P-labeled cDNA probes were prepared from 3 μg total RNA from KM12C, KML4A, and KM20 cells. The probes were hybridized to separate Atlas Human 1.2 cDNA expression array membranes. Results were analyzed by autoradiography, and expression of genes common to all three cell lines was compared. (**B**) Nine genes common to all three cell lines were found to be increased or decreased by more than threefold in KM20 and KML4A compared with KM12C cells.

To focus our study, we determined whether differences in gene expression patterns existed between the more aggressive cell lines (KML4A and KM20) compared with KM12C cells, which are derived from a Dukes B colon cancer. A threefold or greater increase in gene expression is denoted by red; conversely, a decrease in gene expression of threefold or greater is denoted by blue. Genes found to have no difference in expression pattern are shown in green. Nine genes common to both KML4A and KM20 cell lines were found to have a threefold difference in expression pattern (either increased or decreased) (see Fig. 1B). An increase in expression of six genes was identified:

- Zyxin, a gene implicated in several important signaling pathways that regulate cell differentiation, proliferation, and morphology[23]
- FRP1, a member of the PIK-related kinase family known to control cell cycle progression in the presence of DNA damage[24]
- GADD153, a DNA damage-inducible protein[25]

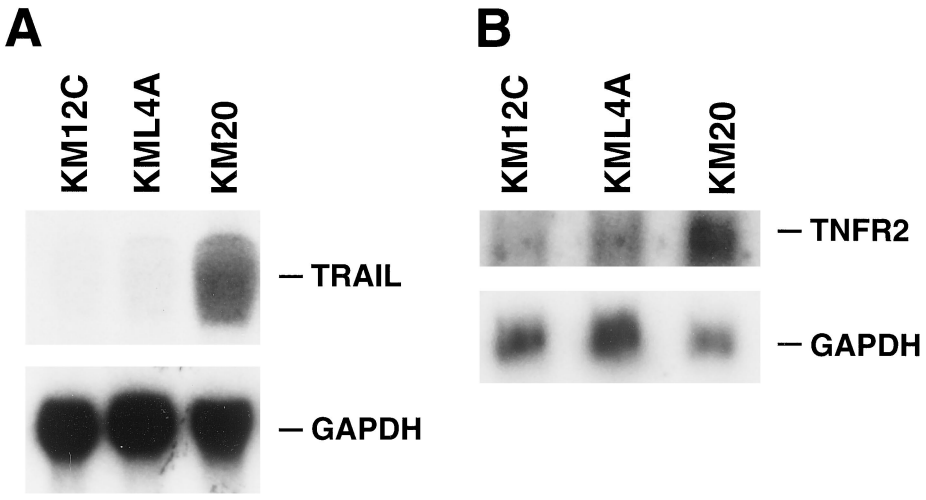

Figure 2. Expression of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and tumor necrosis factor receptor 2 (TNFR2) mRNA. Northern blot analysis of TRAIL (**A**) and TNFR2 (**B**) mRNA expression in KM12C, KML4A, and KM20 cells. To ensure intact RNA and relatively equal RNA loading, the blot was stripped and reprobed with a ^{32}P-labeled human GAPDH probe.

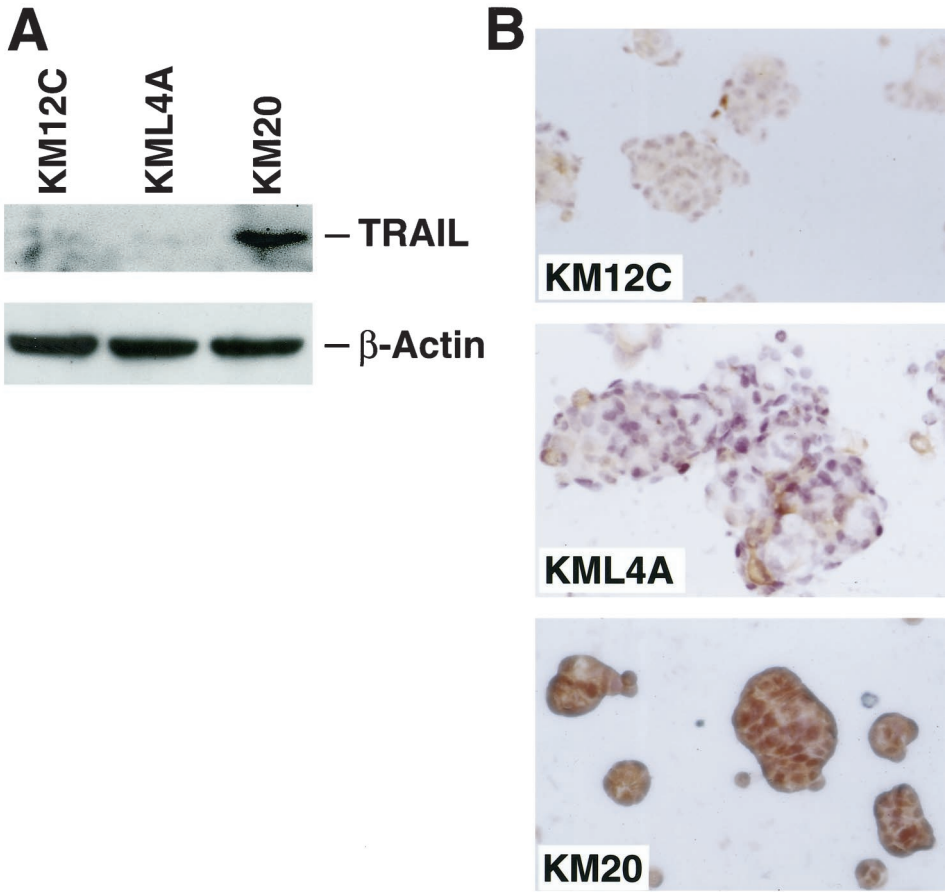

Figure 3. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) protein expression. (**A**) Western blot analysis of protein (100 μg) extracted from KM12C, KML4A, and KM20 cells using a human monoclonal anti-TRAIL antibody. The blot was reprobed with β-actin to normalize for protein loading. (**B**) Immunohistochemical analysis of TRAIL protein expression in the KM cell lines using the human TRAIL antibody as described for the Western blots. TRAIL expression (noted by the brown stain) was most abundant in the KM20 cell line (magnification, ×20).

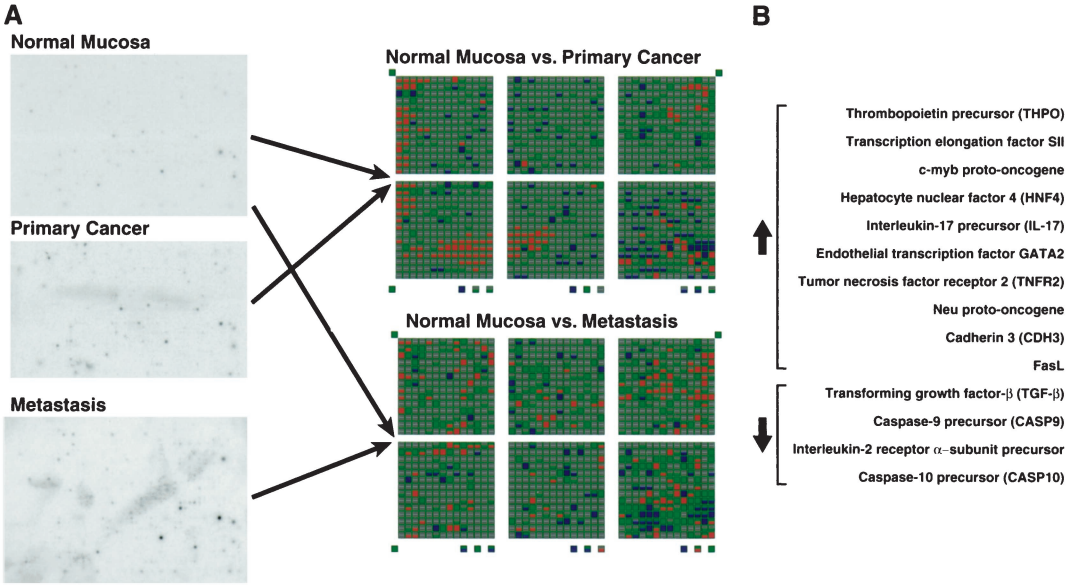

Figure 4. Gene expression profile in vivo. (**A**) ^{32}P-labeled cDNA probes were prepared from 3 μg total RNA from normal colonic mucosa, primary colon cancer, and a liver metastasis in a patient with a Dukes D colon cancer. The probes were hybridized to separate Atlas Human 1.2 cDNA expression array membranes. Results were analyzed by autoradiography, and expression of genes common to all three tissues was compared. (**B**) Fourteen genes were found to be increased or decreased by more than threefold compared with normal mucosa.

- TNFR2, a reported activator of NFκB, which is associated with cell survival[26]
- Junction plakoglobin, a protein involved in cell adhesion[27]
- TRAIL, a novel member of the TNF/NGF family of proteins, which results in the death of activated T cells both in vivo and in vitro by a process of apoptosis.[9]

The remaining three genes all exhibited a reduction in gene expression:

- Acyl-CoA-binding protein, a protein with high affinity for long-chain acyl-CoA esters thought to have a role as a regulator of cellular functions[28]
- 26S protease regulatory subunit: overexpression of this protein has been demonstrated to diminish cell proliferation[29]
- c-ets-2, a common transcription factor that can alter expression of genes important for cell proliferation.[30]

Assessment of TRAIL and TNFR2 Expression in the KM Cell Lines

Our findings by cDNA expression arrays identified potential genes that may be altered with progression to a metastatic phenotype. To confirm the array findings of increased expression of TRAIL and TNFR2, Northern blot analyses were performed (Fig. 2). Expression of TRAIL mRNA was markedly increased in the KM20 cell line compared with KML4A and KM12C cells. Although TRAIL mRNA levels were increased in KML4A compared with KM12C, these changes were not as dramatic as that noted for KM20. These findings emphasize the fact that the cDNA expression array provides only a semiquantitative assessment of gene expression. A definitive assessment of mRNA abundance may be obtained with the Northern blot (see Fig. 2) or alternatively RNase protection studies. In addition, levels of TNFR2 were assessed by Northern blot, with the finding of increased mRNA expression in both the KML4A and KM20 cells compared with KM12C. This is consistent with our results using the cDNA arrays.

As a further assessment of TRAIL expression in the KM cell lines, Western immunoblot analysis and immunohistochemical staining for TRAIL were performed (Fig. 3). Similar to the Northern blot results, TRAIL protein expression was dramatically increased in the KM20 cell line (see Fig. 3A). Moreover, immunohistochemical staining provided further corroboration of a differential expression pattern, with KM20 and KML4A demonstrating increased TRAIL staining (shown by the brown color) compared with KM12C (see Fig. 3B). Collectively, these findings extend and confirm our results using cDNA expression arrays. Further, members of the TNF family of proteins (e.g., TRAIL, TNFR2) may contribute to the metastatic phenotype.

Gene Expression Pattern in Metastatic Colon Cancer In Vivo

To extend our initial findings using in vitro human colon cancer cell lines, we analyzed tissue (normal colonic mucosa, primary colon cancer, and liver metastasis) resected from a patient with Dukes D colon cancer by cDNA expression array (Fig. 4). Of the roughly 1,200 genes assessed, expression of 33 (2.8%), 73 (6.2%), and 116 (9.8%) genes was noted in normal mucosa, colon cancer, and liver metastasis, respectively. The expression of 14 genes was altered more than threefold in the primary cancer and liver metastasis compared with the normal mucosa. Of these 14, increased expression was noted for 10 genes—the c-*myb* and c-*erb*B2 protooncogenes, TNFR2, hepatocyte nuclear factor-4 (HNF-4), the GATA-2 transcription factor, cadherin-3, the transcription elongation factor SII, thrombopoietin precursor, interleukin-17 precursor, and FasL. The remaining four genes demonstrated decreased expression in the primary colon cancer and the liver metastasis—caspase-9 and caspase-10 precursors, interleukin-2 receptor α subunit, and transforming growth factor-β. In all instances, metastasis resulted in a more profound alteration in gene expression patterns compared with the primary cancer. Of particular note is the finding of increased expression levels of TNFR2 and the TNF-like proteins, TRAIL or FasL, in the more metastatic colon cancer cells and the liver metastasis in vivo, further suggesting a role for these proteins in the metastatic phenotype.

DISCUSSION

In this study, we used cDNA expression arrays to analyze changes in the expression patterns of roughly 1,200 cancer-related genes to determine genetic changes that lead to the complex metastatic phenotype. We analyzed both in vitro cell lines of varying metastatic potential as well as in vivo tissue samples from a patient with metastatic colon cancer and identified genes with threefold or greater changes in gene expression levels. In particular, we showed that TNF family proteins (i.e., TRAIL/FasL, TNFR2) may play an important role in metastasis through an "immune escape" phenomenon, which allows cancer cells to evade immune detection. The ability to analyze and compare the expression patterns of multiple genes simultaneously provides a powerful technique to identify potential molecular targets for novel therapeutic strategies.

The large-scale sequencing efforts derived from the ongoing Human Genome Project has resulted in the identification and partial sequence analysis of thousands of genes.[31] With the identification of these genes, a new technology has emerged that will allow us to understand the role of these genes in both normal and disease states. This approach involves the hybridization of entire cDNA populations to nucleic acid arrays, thus allowing high-throughput analysis of the expression patterns of multiple genes simul-

taneously.[32] This technique has a wide range of applications, including the assessment of differential gene expression patterns associated with normal and pathologic conditions.

In our study, we used cDNA expression arrays to analyze changes in gene expression in human colon cancers of differing metastatic potential. We identified a more than threefold alteration in the expression of nine genes in the KML4A and KM20 cell lines compared with KM12C, which was derived from a patient with Dukes D colon cancer. Increased gene expression was demonstrated for Zyxin, FRP1, GADD153, and junction plakoglobin. Zyxin, a gene implicated in cell differentiation and proliferation, has gained recent attention for its role in cell motility, which may provide for increased neoplastic cell proliferation and migration.[23] FRP1 is a protein involved in the regulation of the cell cycle after DNA damage.[24] Mutant forms of FRP1, which are not capable of stabilizing damaged DNA, may lead to chromosomal instability in the development of cancer. The transcription factor GADD153 (CHOP) is known to heterodimerize with C/EBP family members, thus preventing their binding to DNA sequences.[25] These CHOP-C/EBP heterodimers may bind to alternative DNA sequences and regulate the transcription of other genes important for cancer cell progression, such as protooncogenes or cell survival mediators such as FasL and TRAIL. Junction plakoglobin, similar to β-catenin, is known to bind to the tumor suppressor gene APC; therefore, junction plakoglobin may have similar interactions in the regulation of gene transcription as β-catenin. [27] The expression of three genes (acyl-CoA-binding protein, 26S regulatory subunit, and the transcription factor c-ets-2) was noted in KML4A and KM20 cells compared with KM12C. Acyl-CoA-binding protein binds long-chain acyl-CoA with high affinity and is thought to play an important role in intracellular acyl-CoA transport.[28] The reduction in the expression of this protein may lead to unexpected derangements in cellular processes leading to the formation of neoplastic cells.[28] The 26S protease regulatory subunit degrades ubiquitinated proteins and is essential for a wide range of cellular processes, such as cellular proliferation.[29] Overexpression of this protein is thought to have a negative effect on cellular proliferation; therefore, the reduced expression noted in the more metastatic cells may offer a proliferative advantage. Taken together, these KM cell lines represent useful in vitro models to elucidate mediators that may contribute to the metastatic phenotype in colon cancer cells.

To characterize the roles of genes that were noted to be increased in the more metastatic KM cell lines, we focused on two members of the TNF family (TRAIL and TNFR2), both of which were increased in KML4A and KM20 cells compared with KM12C cells. TRAIL is a novel membrane protein that, like FasL, is a membrane protein capable of inducing apoptotic cell death in various cell types.[9] Although normal tissues appear resistant to TRAIL treatment, TRAIL has been shown to induce the death of activated leukocytes.[8,9] Therefore, similar to FasL, this protein may play an important role in the escape of certain cancer cells from surveillance and may contribute to the metastatic phenotype. Increases in TRAIL mRNA and protein were demonstrated particularly in the KM20 cell line, which is consistent with our results using the cDNA expression arrays. Future studies will be able to delineate whether this increase in TRAIL expression produces functional consequences in these colon cancer cells, such as increased T-cell death. In addition to TRAIL, increased expression was noted for TNFR2 in the more metastatic KM cell lines; this was confirmed by Northern blot analysis. TNFR2 is a protein implicated in both cell death and cell survival,[26] but its role in colorectal cancer has not been previously defined. Interaction of TNFR2 with its ligand (TNF) has been reported to activate the transcription factor NFκB, which is associated with cell survival in various cell types.[26] Therefore, increased TNFR2 expression may confer a survival advantage to the more metastatic KM cell lines.

We next assessed changes in gene expression patterns in vivo using resected tissue samples from a patient with metastatic colon cancer. We compared the expression patterns of normal adjacent colonic mucosa, primary colon cancer, and liver metastasis and detected a more than threefold change in the expression of 14 genes. Ten genes were noted to be increased in the primary colon cancer and liver metastasis compared with normal colonic mucosa. Genes that demonstrated increased expression, favoring cell survival and proliferation, included the protooncogenes c-*myb* and c-*erb*B2.[31,32] In addition, the transcriptional regulators HNF-4, GATA-2, transcription elongation factor S2, and transforming growth factor-β were increased in the primary and metastatic cancer tissues.[33–36] Increased expression of cadherin was also noted, which may provide an important element in the progression of primary tumors to the development of distant metastases.[34] Consistent with our in vitro findings, we also noted increased expression of TNFR2 in the primary cancer and hepatic metastasis in vivo. In addition, FasL, a TNF family protein with functions similar to those of TRAIL, was increased in the cancer and liver metastasis. Similar to TNF, FasL induces apoptosis in many cell types, thus providing immune privilege for certain tissues of tumors from immune detection.[8] Taken together, the findings of increased expression of TRAIL and FasL, as well as TNFR2, identify these proteins as potentially important contributors to colorectal cancer metastasis.

We also identified other genes with a graded increase or decrease in gene expression when comparing normal mucosa with the primary tumor and, ultimately, with liver metastasis. These genes may further contribute to the overall metastatic phenotype. However, additional work, including an analysis of expression patterns in other patients with metastatic colorectal cancer, is clearly needed to confirm these initial findings. With the analysis of additional colorectal cancers (both metastatic and nonmetastatic), the expression patterns of genes that contribute to a metastatic

phenotype may be better characterized. Therefore, in the future, the likelihood of tumor cells to metastasize may be assessed using gene expression profiles.

In conclusion, we examined the gene expression patterns of colon cancer cells and in vivo tissues using cDNA expression arrays. Genes with altered expression patterns were identified in the more metastatic KM cell lines and in the progression from normal to primary cancer to liver metastasis in vivo. Increased expression of members of the TNF family of proteins was common to both the in vitro and in vivo analyses. These proteins included TRAIL (in vitro), FasL (in vivo), and TNFR2 (both), thus suggesting a role for these proteins in colon cancer metastasis. Additional studies are required to confirm and extend these initial results and to delineate the functional contributions of these proteins to the overall metastatic phenotype. Using novel techniques such as cDNA expression arrays will lead to a better understanding of the molecular mechanisms regulating colon cancer metastasis. This analysis may provide for a method for characterizing cancers based on their gene expression pattern to predict a more clinically aggressive tumor type. Identification of altered gene expression patterns may lead to the development of novel therapeutic strategies that can be used as adjuvant therapies in the treatment of colorectal cancers.

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Discussion

DR. YUMAN FONG (New York, New York): Microarray hybridization analysis certainly represents a potentially very important method for studying human disease. It allows screening of small samples for many, many genes and therefore allows us potentially to examine not only mechanism of disease, but screen for targets for therapeutic modalities. But there are certain pitfalls, too. Microarray hybridization is a big fishing exercise. And it is molecular fishing, which is more expensive than regular fishing. So I congratulate the authors in not only finding genes that may be different in metastatic tumors but also going beyond this and formulating a hypothesis for how these genes may behave. Some questions, though.

First, the authors attribute their gene differences to the metastatic potentials of the three different cell lines that they looked at. But it may be related to other characteristics of the cell lines, too. The zysome gene is related to proliferation, the GADD genes are DNA repair genes, and certainly the TNF-related family genes can also relate to proliferation. Can the authors describe to us the various cell lines in terms of S-phase fractions, doubling times, and other characteristics that may assure us that the hybridization studies they have done are more than an expensive measurement of proliferation and mitotic rate?

Second, not all tumor metastases are made the same. We know from examining lung and liver metastases, for example, that there are a lot of cellular differences even in the same patient for tumors that have gone to the lung and to the liver. A lot of the cellular differences relate to proliferation and cellular pathways for DNA synthesis. Have the authors looked at a comparison of lung/liver metastases or tumors from different parts of the body to tell us whether such hybridization studies in the genes that they are looking at may be different depending on where the tumor ends up in vivo?

Third, have the authors looked at more samples besides the first very encouraging patient to confirm the finding that the TNF family genes may be related to the metastatic potential?

Fourth, can the authors tell us how much it costs approximately to do that one single patient so that we can put it in perspective in terms of long-term screening for patients?

Lastly, I just want to put in one more plug for the American College of Surgeons Oncology Group. Studies like this for microarray hybridization are perfect for cooperative groups, where we can get many surgical samples, look in a screen for many genes, and also have an infrastructure to analyze that data to make heads or tails of it. Therefore, in all the studies we are thinking of for the American College of Surgeons Oncology Group, we should think of correlative studies that may use technology such as the microarray hybridization.

PRESENTER DR. B. MARK EVERS (Galveston, Texas): I appreciate your comments and totally agree with the fact that currently we are performing a molecular fishing expedition; however, I do think that these studies are important to determine the potential proteins which may be contributing to metastatic disease. After this initial survey, it is then incumbent on the investigator to identify those genes which appear most promising and perform further analyses so as to confirm the changes noted in gene expression, and then ultimately to perform functional studies to determine the significance of these changes in metastatic tumors. Currently, we are evaluating the TNF family members, since our studies, to date, have identified that these proteins may be important with regard to colorectal cancer metastasis. We have preliminary data to show that in the more metastatic cell lines, KM20 and KML4A, there is increased T-cell death when cocultured with these tumor cells, suggesting an increased killing capacity for these more aggressive cell lines which would correlate with our findings by gene array.

You asked a question regarding the cell lines that we have utilized in our studies. These cell lines, developed by Dr. Isaiah Fidler at M.D. Anderson, provide an attractive model to evaluate tumor characteristics both in vitro and in vivo. The more metastatic cell lines mimic the clinical situation with metastasis noted to the liver when these cells are placed either in the spleen or the cecum. In addition, the KML4A cell line is actually derived from KM12C cells; therefore, the genetic background should be relatively the same except for its propensity to metastasize.

You asked about changes in metastatic patterns—that is, liver metastasis versus lung metastasis. We have not specifically looked at patients with lung metastasis yet. However, as we broaden the study, this is certainly the plan to accumulate as many patients as possible to evaluate both the primary as well as the metastatic lesions. Also, we are currently evaluating the use of laser capture microdissection to more precisely ensure that only tumor cells are examined and not surrounding normal tissue. This technique will greatly add to our studies.

You asked whether we have looked at other TNF receptor family members to determine whether they may be involved in the process of metastasis. These are studies that we currently have ongoing and, based upon additional information from more tumor samples, we may, hopefully, be able to provide answers to these questions.

You asked about the cost of doing this one study, since, as you have alluded to, these cDNA arrays are quite expensive. This one study cost between $4,000 and $5,000. Within the next 5 to 10 years, however, these costs will decrease and, in fact, one can envision that in the future we will be ordering cDNA expression arrays on our patients similar to how we routinely obtain blood and electrolyte analyses.

DR. MARSHALL M. URIST (Birmingham, Alabama): I would like to know about the expression of TNF family of proteins. Is there any other information related to using these to predict sensitivity of adjuvant therapies? And what is different about this approach to identify these proteins? We have made very little progress in the gene therapy of cancer. What is different about these compared to other genes that are currently being targeted for gene therapy?

DR. EVERS: Dr. Urist, you asked about the TNF family members, and what makes these proteins different is the fact that they are involved in the process of immune privilege, for example. So, it is felt that these proteins, which include FasL and TRAIL, can confer a survival advantage to cancer cells or, for that matter, certain normal cells. In addition to producing these proteins, we also have preliminary data to show that these colon cancer cells are actually resistant to TRAIL treatment, which confers an additional advantage to these cells for eventual metastasis. Obtaining information on a number of different colon cancers and metastatic lesions from different patients will allow us to better determine the overall importance of the TNF family of proteins in this process. It is then hoped that therapies can be developed as adjuvant treatment for colorectal cancer metastasis based upon this information.

DR. JOHN S. SPRATT (Louisville, Kentucky): My experience in this goes back some 30 years, when we reported a large series of cases, over 600, clearly demonstrating that the probability of metastasis of the colon cancer is completely independent of the size of cancer up to cancers 15 to 20 cm in size (*Dis Colon Rectum* 1970;13:243–246). This is not made obvious by the TNM system.

We knew that there was an underlying molecular biological and genetic basis for this, but we weren't smart enough to figure it out. We later did it in more detail using the analysis of variance and covariance so we could actually identify what characteristics of patient and cancer were contributing to mortality (*J Surg Oncol* 1976;8:155–163).

However, the main value of this study from my perspective is the fact that you can identify on a molecular biological basis of these metastasizing and nonmetastasizing subsets. If you demonstrate or confirm a nonmetastasizing subset, those patients don't need a lot of high-cost chemotherapy and radiation therapy as an adjuvant adding to the morbidity and cost of the disease, so you can more specifically target definitive surgical therapy toward nonmetastasizing cancers that are locally curable.

DR. EVERS: Dr. Spratt, I couldn't agree with you more, and I really appreciate your comments. The work that we are presenting today was predicated by our clinical experience in patients with colorectal cancer. I think that we have all seen patients with large bulky tumors that have not metastasized. Conversely, we have also seen relatively small tumors that have metastasized widely to the liver and to the lungs. Therefore, I believe that this process is based upon the genetic makeup of these particular tumors, and, as you alluded to, knowing information from studies such as these may allow us to better tailor the adjuvant therapy to the particular genetic makeup of these cancers.

DR. DAVID ALLISON (Toledo, Ohio): The authors are to be congratulated on their attempt to find genetic determinants of tumor behavior. I think there is an elegant underlying hypothesis for this work, being that individual cancers have undergone different permutations of their genomes which, in turn, lead to varying clinical outcomes.

These genetic changes could potentially reside in the primary base sequence of the tumor DNA, the ordering of gene arrays and controlling elements, changing in the binding of histones and DNA-associated proteins in the chromatin, changes in DNA methylation patterns, and possibly even from epigenetic silencing. The tumor-specific genetic changes could lead to changes in mRNA transcription, translation, or in posttransitional protein modifications involved in cellular signaling cascades. I think this paper is a very good start to address these questions at the transcriptional level.

I have a few questions for the authors which may simply be ideas for future work. Do you have correlates of the cell cycle parameters of your cell lines, such as doubling times or the percentage of cells in S phase, with specific patterns of gene expression? Have you performed in vitro invasion assays to see whether or not the results of such tests correlate with expression of the putative invasive genes? Perhaps most importantly, do these lines show either microsatellite instability or chromosomal abnormalities secondary to frank aneuploidy? If so, do the patterns of gene expression spontaneously shift over time, or in response to perturbations of the culture system?

DR. EVERS: Dr. Allison, I appreciate your thoughtful comments. We have not yet looked at some of the functional assays that you have mentioned. Obviously, this represents the next set of investigations to perform. We do plan on expanding these studies and using arrays with a larger set of genes, including the oncogenes and tumor suppressor genes that you have alluded to. This represents a powerful technique; however, these studies are very much in their infancy and will require analysis of a number of cancers from different patients before any definitive statements can be made. I appreciate, once again, the thoughtful comments of all of the discussants.